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EFFECT OF DIETARY SUPPLEMENTATION WITH α-TOCOPHERYL ACETATE AND ASCORBIC ACID ON QUALITATIVE CHARACTERISTICS AND FERTILIZING ABILITY OF RABBIT SEMEN

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ABSTRACT : The aim of the present work was to verify the effect of α-tocopheryl acetate addition - 50 vs 200 mg kg^{-1} - and ascorbic acid - 0 vs 1 mg L^{-1} - on the characteristics of rabbit semen and its fertilizing ability. The single supplementation of vitamin E and C, compared to the control diet, did not modify semen traits, while the association of E+C significantly improved the kinetics of spermatozoa (60.73 vs 57.09 μm sec^{-1} - path velocity) but without any effect on the fertility rate (70.00 vs 70.66 %). The results confirm that the standard recommendations for vitamin E and C are adequate if fresh semen is used.

RÉSUMÉ : Effets de l'acétate d'α-tocopherol et de l'acide ascorbique ajoutés à l'alimentation sur la qualité et le pouvoir fertilisant du sperme de lapin.
Le but de ce travail est de vérifier l'effet de l'addition d'acétate d'α-tocopherol dans l'aliment – 50 *vs* 200 mg/kg^{-1} – et de l'acide ascorbique dans l'eau de boisson – 0 *vs* 1 mgl^{-1} – sur les caractéristiques du sperme de lapin et son pouvoir fertilisant. Comparée au régime témoin, la supplémentation par les vitamines E et C seules, ne modifie pas les caractéristiques du sperme, tandis que l'association E+C augmente significativement la cinétique des spermatozoides (60,73 *vs* 57,09 μm sec^{-1} – vitesse d'acheminement) sans aucun effet sur le taux de feritlité (70,00 *vs* 70,66 %). Ces résultats confirment que les recommandations habituelles pour les vitamines E et C sont adéquates lorsque la semence est utilisée fraîche.

INTRODUCTION

Artificial insemination (A.I.) requires spermatozoa with excellent qualitative characteristics especially if insemination is performed with refrigerated or frozen semen. In rabbit, the factors affecting fertility of the doe have been quite well investigated, while only a few studies have been done on bucks

Regarding the nutritional requirement of males, some researchers have analyzed the role of certain antioxidants on the characteristics of semen in boar (MARIN-GUZMAN *et al.*, 1997) and cock (SURAI *et al.*, 1997). It has been shown that vitamin E plays an important protective action on the membrane integrity and lipid stability of both the seminal plasma and spermatozoa (BRZEZINSKA-SLEBODZINSKA *et al.*, 1995, TEROND *et al.*, 1996).

The high degree of unsaturation of mammalian sperm lipids (POULOS *et al.*, 1973) renders these gametes very susceptible to peroxidation which in turn has been proposed as one of the major causes of male infertility (JONES *et al.*, 1979, MANN and LUTWALK-MANN, 1981).

Since in commercial rabbitries considerable A.I. is done with fresh semen directly collected and diluted by the breeder, the present work investigated the effect of vitamins E and C on the qualitative characteristics of rabbit semen and its fertilizing ability.

MATERIALS AND METHODS

The trial was carried out from May to December 1998 in the experimental rabbitry of the Animal Science Department. The environmental temperature ranged from 25.0 to 31.0° ± 3.4 C and the photoperiod was 16 hours light/day.

Sixty, 5-month old N.Z.W. bucks were divided into 4 homogeneous groups and were fed *ad libitum* two diets containing, respectively, 50 (control) or 200 mg kg^{-1} of α-tocopheryl acetate (vitamin E) (Table 1). Vitamin C was administered by water (0 vs 1 g L^{-1}).

The groups were the following:
- Control (50 mg kg^{-1} α-tocopheryl acetate);
- vitamin C (1 g L^{-1} vitamin C);
- vitamin E (200 mg kg^{-1} α-tocopheryl acetate);
- vitamins E+C (200 mg kg^{-1} α-tocopheryl acetate + 1 g L^{-1} vitamin C).

The chemical composition of the diets was determined according to AOAC (1995). The α-tocopherol level in diets was quantified by HPLC (ZASPEL and CSALLANY, 1983). Fatty acids of diets and meat were extracted according to FOLCH et *al.* (1957) and determined as methyl esters with a gas chromatograph using a D-B wax capillary column (25 mm ∅, 30 m length). The major fatty acids of diets were the following 20.91% of saturated fatty acid, 23.04% of mono-unsaturated, 56.05% of PUFA (44.18 and 9.39 % of linoleic and linolenic acid, respectively).

After an adaptation period of 1 month, semen was collected and analyzed weekly for a 27-week period. It was collected by artificial vagina and samples were maintained at room temperature and analyzed within 1 hour. Sperm concentration was estimated by a Thoma-Zeiss cell chamber (final dilution 1:100) and kinetic parameters by CASA (Computer-Assisted Sperm Analysis). Aliquots (10 μl) of the samples diluted 1:30 with Tris-glucose-citrate (300 mOsm g^{-1}, pH 7.1) were laid over a pre-warmed Makler chamber at 37 °C and 6 fields (2 drops x 3 fields) were analyzed by CASA (SCA 3.0, Microptic, Spain) using set-up parameters (Table 2) previously established for better

Table 1 : Formulation (%) and chemical composition of diets

Dehydrated alfalfa meal	40
Barley meal	32.5
Soybean meal	19
Wheat straw	4
Molasses	0.8
$CaCO_3$	1
Calcium diphosphate	0.6
Ligninsulfunate	0.5
Salt	0.5 *vs* 0.4985
Coccidiostat	0.06
DL-Methionine	0.04
Vitamin-mineral premix*	1
α-tocopheryl acetate	**0 *vs* 0.0015**
% dry matter :	
Crude protein	*17.60*
Ether extract	*2.83*
Crude fibre	*17.65*
Ash	*8.60*
N-free extract	*53.28*
NDF	*30.10*
ADF	*20.15*
ADL	*3.75*
Hemicellulose	*9.95*
Cellulose	*15.48*
mg kg^{-1}	
α-tocopherol	85 *vs* 255

* Added per kg: Vit. A 11,000 UI; Vit. D_3 2,000 UI Vit. B_1 2.5 mg; Vit. B_2 4 mg; Vit. B_6 1.25 mg; Vit B_{12} 0.01 mg; **α-tocopheryl acetate 50 mg**; Biotin 0.06 mg; Vit. K 2.5 mg; Niacine15 mg; Folic aci 0.30 mg; D-pantothenic acid 10 mg; Choline 600 mg Mn 60 mg; Fe 50 mg; Zn 15 mg; I 0.5 mg; Co 0.5 mg

Table 2 : Parameter setting used for rabbit semen

Frame at frame rate		16 to 25/second
Minimum contrast		130
Cell size (min-max)	μm²	10.5-24
Shape	Perimeter²/ 2 x cell size	0.5-5
Minimum data point		8
Low VAP	μm s^{-1}	20
Medium VAP	μm s^{-1}	40
Threshold straightness	%	80

Table 3 : Significance of effects and R^2

	Treatment	Treatment x week of collection	Buck (treatment)	R^2 (%)
Volume	n.s.	**	**	25
Number of spermatozoa	n.s.	**	**	35
Live cells	n.s.	**	**	42
Motille cells	n.s.	**	**	41
VAP	**	**	**	35
VSL	**	**	**	34
VCL	**	**	**	22
LIN	*	**	**	31
ALH	**	**	**	21
Fertility rate	n.s.	-	-	-

n.s = P > 0.05; ** = P < 0.01

discriminating granules from static cells (LATTAIOLI and CASTELLINI, 1998). Recorded sperm parameters were: motility percentage, progressive speed (VSL μm s^{-1}), track speed (VCL μm s^{-1}), path velocity (VAP μm s^{-1}), linearity (LIN = VSL/VCL x 100) and ALH (amplitude of lateral head displacement).

After the 27-week period, in two successive times, 1,200 multiparous does (300 per treatment) were inseminated with about 10 million motile spermatozoa per doe (CASTELLINI *et al.*, 1999). No estrus synchronization was done and ovulation was induced by inoculating 20 μg of GnRH per doe.

Data were statistically evaluated with a complete factorial model (treatment, week of collection x treatment, buck within treatment) for repeated measurements. Several procedures of the SAS library were used (SAS, 1990 - GLM for linear models, CATMOD for fertility rate).

RESULTS

Diets

The dietary levels of α-tocopherol were 85 and 255 mg kg^{-1} diet. Thus, vitamin E was higher than recommendations (50 mg kg^{-1} - INRA, 1989) also in the control diet (Table 1).

Semen quality

The significance of the effects and the adequacy of the model are presented in Table 3. Differences between experimental groups were significant except for volume, concentration, live sperm cells and motility rate; the other effects (week of collection x treatment and buck within treatment) significantly influenced all the parameters. The ALH, VCL and volume had the lowest R^2 (21-25%), while it was above 30% for the other semen traits.

The supplementation of a single vitamin did not improve any spermatozoa characteristic (Table 4) and vitamin E alone reduced VSL and LIN. On the contrary, the combination of ascorbate and α-tocopherol increased kinetic parameters (VAP, VCL and ALH); however, such improvements did not affect the fertilizing ability of the fresh semen.

The effect of treatments during the 27 weeks of semen collection is shown in Figures 1 and 2 for sperm motility and track speed, respectively.

The track speed, which outlines other kinetic components (VCL, VSL), showed an increase related to the length of administration in the group fed the combination of the two vitamins.

DISCUSSION

The α-tocopherol levels of the diets was 1.7 to 5.1 times higher than recommendations and indicated a good resistance of α-tocopheryl acetate to feed processing.

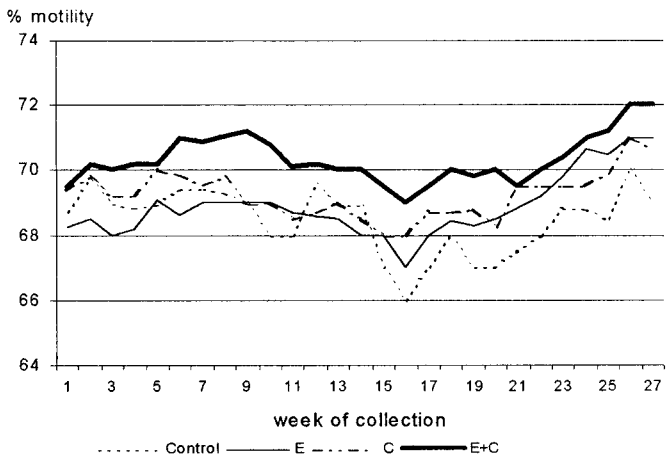

Figure 1 : Effect of the week of collection and treatment on the motility rate of spermatozoa

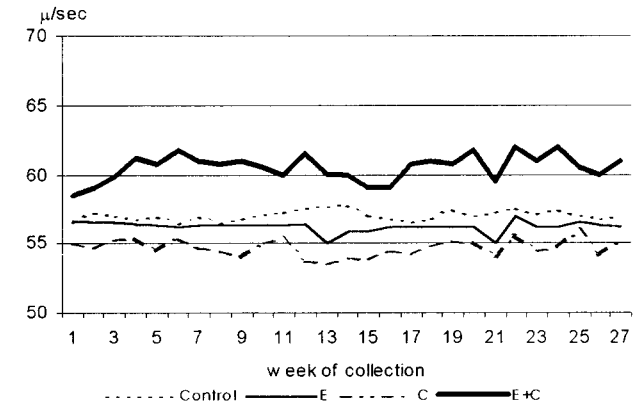

Figure 2 : Effect of the week of collection and treatment on the path velocity of spermatozoa

The percentage of motile cells, path velocity and linearity were in agreement with findings obtained with HTM-IVOS by FARREL *et al.* (1993) and THEAU-CLEMENT *et al.* (1996) at a similar dilution rate. Later, FARRELL *et al.* (1996), analyzing less diluted sperm samples containing about 25 x 10^6 cells, reported a higher velocity and ALH. These discrepancies could be ascribed to the negative effect of excessive dilution on the viability of cells and on their motility (FARRELL *et al.*, 1996).

Since CASA estimations are mainly used to predict the fertilizing ability of semen, a dilution similar to the one used under field conditions for A.I. (about 25-30 fold, CASTELLINI *et al.*, 1999) should be appropriate and could be used as a reference for rabbit.

Further differences could be due to the individual variation of the bucks, the different model of CASA (JASKO *et al.*, 1990) and the conditions of analysis (medium, number of drops and fields).

Regarding the effect of vitamins, our findings partially agree with those of EL-MASRY *et al.* (1994) who did not find a positive effect of dietary vitamin E (40 mg kg^{-1}) and selenium (0.7 mg kg^{-1}) on spermatozoa motility and the reproductive performance of rabbit bucks, but only on the sperm concentration. Also, MINELLI *et al.* (1999), by feeding young bucks (from 20 to 23 weeks of age) with supranutritional levels of vitamin E (400 mg kg^{-1}) and vitamin C (1 g L^{-1}) did not find significant improvements in semen parameters; probably the short duration of the trial did not give enough time to show differences. In our study, differences were greater when the supplementation period was longer than 5 weeks (Figure 2).

The oral supplementation of vitamin E has appeared effective only when compared with deficient diets (MARIN-GUZMAN *et al.*, 1997) or when men with low fertility rates have been treated (GEVA *et al.*, 1996).

The failure of the single addition of vitamin E is probably related to the partial resistance of the semen to modify its basal level of α-tocopherol (SURAI *et al.*, 1997). In men, MOILANEN and HOVATTA (1995), administering different doses of α-tocopherol, showed a non-linear augmentation of its level in seminal plasma (0.1-0.5 fold) below 1 µmol L^{-1} which was found effective in protecting spermatozoa from peroxide damage. Improvements in fertilizing ability of semen can be achieved only when the level of vitamin E in the plasma and in the cells are much higher (from 2 to 5 fold) than the standard level (BLESBOIS *et al.*, 1993).

Although in our assay we did not analyze the susceptibility of the semen to oxidation, the positive effect of the simultaneous administration of vitamins E and C on spermatozoa kinetic parameters was presumably due to the sparing effect of ascorbate on vitamin E (NIKI, 1984) which increases the viability of cells by preserving bio-membranes from lipid peroxidation

Table 4 - Effect of α-tocopherol and ascorbic acid on various semen parameters and reproductive performance (n = 1,780)

		Control	Vitamin E	Vitamin C	Vitamins E + C	Pooled s.e.
Volume	ml	0.55	0.58	0.60	0.55	0.10
Number of spermatozoa	n. x 10^6 ml^{-1}	395.4	409.5	405.1	410.1	137.4
Live cells	%	81.51	82.50	82.98	84.00	22.50
Motile cells	"	68.51	68.86	69.16	70.23	15.61
VAP	μsec^{-1}	57.09A	55.93A	54.02A	60.73B	13.93
VSL	"	41.70B	39.96A	38.90A	41.26B	15.32
VCL	"	95.59A	95.03A	91.25A	103.00B	26.36
LIN	%	44.00b	41.32a	42.50ab	40.26a	15.64
ALH	µm	3.54AB	3.62B	3.33A	3.91C	1.92
Fertility rate	%	70.00	72.33	68.33	70.66	7.57*

On the same row : a..b : P < 0.05 ; A.C: P < 0.01. * Chi square value

(NUNES *et al.*, 1995). Such improvement, however, did not affect the fertilizing ability of semen; a different hypothesis could be developed to explain this lack of correlation:

- the differences in motion traits, although significant, were not sufficiently relevant to affect fertility rate;
- the number of sperm for A.I. used (10^6 motile sperm/A.I.), although assesed to assure suitable reproductive performance (CASTELLINI *et al.*, 1999), may be too high to detect small differences in sperm quality;
- fresh semen had a low peroxidation process and even the lowest antioxidant strength of the control diet was probably enough to control it. Additional levels of these vitamins, enhancing the resistance of cells to peroxidation (ASKARI *et al.*, 1994), could be more effective if spermatozoa must be processed. BLESBOIS *et al.* (1993), adding vitamin E to the storage diluent, found a significant improvement in the fertilizing ability of chicken semen stored for 1 day at 4°C.

The simultaneous administration of vitamins E and C significantly improved the kinetics of spermatozoa, while the single supplementation was ineffective. This improvement however did not correspond to higher fertility.

Our results confirm that standard recommendations for vitamin E and C are adequate if fresh semen is used.

Further studies concerning the effect of the two vitamins on spermatozoa survival after processing (refrigeration, freezing) and their fertility rate should be performed in order to verify if it improves semen quality and fertility rate.

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